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The Antiproliferative and Differentiating Effects of Human Leukemic U937 Cells Are Mediated by Cytokines from Activated Mononuclear Cells by Dietary Mushrooms

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Proliferation of human leukemic U937 cells was remarkably inhibited by conditioned medium (CM) of human peripheral blood mononuclear cells (MNC-CM) stimulated with cold-water extracts (CWE) (10−800 μg/mL of medium) of dietary mushrooms, *Hypsizigus mamoreus* (HM), *Agrocybe aegerita* (AA), *Flammulina velutipes* (FV), whereas insignificant results were observed when cells were cultured in the presence of CWE at the corresponding level. Water extracts from mushrooms were fractionated by Sephadex G-50 chromatography, and the pooled high molecular weight fraction (F1) (200 μg/mL) of HM (HM1) and AA (AA1) exhibited growth inhibitions >80% on U937 cells. Interestingly, the thus-cultured U937 cells showed high nitroblue tetrazolium (NBT) positive (>68%) and nonspecific esterase (NSE) positive (>47%) percentages, revealing the remarkable differentiation into monocytes/macrophages upon incubation with HM1- and AA1-stimulated MNC-CM. In addition, assays for the expressions of monocyte-associated antigens, CD11b, CD14, and CD68, also evidenced the remarkable differentiation of U937 cells into monocytes/macrophages by presenting high CD maker positive percentages (>50%). Tumor necrosis factor (TNF)-α and interleukin (IL)-1β in CM of HM1-stimulated MNC for 1 day (MNC-CM-1) were 1350 and 1374 pg/mL, respectively, revealing the potent antitumor and differentiation-inducing activities of HM. Of note, MNC-CM-1 appeared to be more effective than day 5 MNC-CM (MNC-CM-5) in both antitumor and differentiation-inducing activities.

KEYWORDS: Antiproliferation; differentiation; leukemic U937; conditioned medium; mushrooms

INTRODUCTION

Bioactive components in mushrooms have been the focus of recent research on antitumor, immunomodulatory, antiviral, and antibacterial actions. The bioactive compounds in mushrooms have been indicated to be terpenoids, steroids, phenols, nucleotides, polysaccharides, and proteins (*1*−*3*). Leung et al. (*4*) isolated β-(1→3)-D-glucan of 200 kDa from *Flammulina velutipes* (FV), which displayed 20% growth inhibition on sarcoma SC-180 in vivo. Besides, isolated polysaccharide-rich fractions from FV showed a strong antitumor effect (*5*), whereas the glycoprotein fraction from the same mushroom was proven to be effective in extending the lifespan of B-16 mice implanted with B-16 sarcoma and Ca-755 by 86 and 84%, respectively (*6*). Lectin, a glycoprotein, isolated from *Pleurotus ostreatus* (PO) inhibited growth of sarcoma S-180 and heptoma H-22 by 88 and 75%, respectively (*7*). Therefore, on the basis of the above findings, use of dietary mushrooms as an alternative cancer therapy might be an effective strategy in the treatment of cancers.

The presence and level of cytokines in conditioned medium of mononuclear cells (MNC-CM) upon stimulation during incubation with bioactive ingredients of mushrooms (*8*−*11*) and herbal fungi (*12*) have become interesting subjects of functional food and medicinal herb research. The polysaccharide-rich fraction from fruiting bodies of *Ganoderma lucidum* (*9*) and fu-ling (*Poria cocos*) (*12*) showed potent antiproliferation and differentiation of human leukemic cells through the use of MNC-CM. In addition, the thus-obtained MNC-CM from polysaccharide-stimulated macrophages, T-lymphocytes, or total mononuclear cells (MNC) was assayed to contain high levels of antitumor mediators such as interleukine (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 and, subsequently, led to apoptosis of tumor cells (*9*). Similar results were observed when MNC was treated with the polysaccharide-rich fraction from *Cordyceps sinensis* (*10*). The releases of IL-1β, interferon (IFN)-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) from stimulated MNC by polysaccharides or proteins has been considered as the cell-mediated immunity enhancement (*8*, *9*). Of note, use of MNC-CM usually presents remarkable

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growth inhibition on cancer cells, whereas the direct treatment of cells in culturing medium containing ingredient at the corresponding level displays only insignificant antitumor effect (*9*−*11*).

However, herbal fungi such as *G. lucidum* and *C. sinensis* are costly and, most importantly, hard to obtain; thus, investigations on dietary mushrooms with similar physiological functions appear to be beneficial. To evaluate the antiproliferation and differentiation of U937 cells, cold-water extracts (CWE) from dietary mushrooms were prepared to stimulate peripheral blood MNC for the collection of MNC-CM, with which U937 cells were incubated to observe the changes in cell proliferation, cytoplasmic superoxide production, and nonspecific esterase (NSE) positive percentage. Then, assays for the expression of monocyte-associated antigens in differentiated U937 cells were conducted. Finally, the antitumor mediators in MNC-CM were quantified by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Reagents and Tumor Cell. Ficoll-Hypaque solution (1.077 g/mL) and Sephadex G-50 (1.5−30 kDa for globular protein) were the products of Pharmacia Fine Chemicals (Uppsala, Sweden). Glutamine and RPMI 1640 medium were from Gibco BRL (Gaithersburg, MD), whereas fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Human leukemic U937 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Phytohemagglutinin (PHA), NBT, and NSE kit were from Sigma (St. Louis, MO). A rubber policeman was the product of Bellco Glass (Vineland, NJ). Primary mouse anti-human CD11b, CD14, and CD68 antibodies were from Serotec (Oxford, U.K.). Fluorescein isocyanate (FITC)-conjugated goat IgG fraction to mouse IgG was from ICN (Costa Mesa, CA). Commercial kits for TNF-α and IL-1β quantification in MNC-CM were from R&D (Minneapolis, MN).

Preparation of CWE from Mushroom. Fresh dietary mushrooms of *Hypsizigus marmoreus* (HM), *Agrocybe aegerita* (AA), or *Flammulina velutipes* (FV) were purchased from a local supermarket, and their scientific classification was determined by two fungologists in the Department of Horticulture, National Taiwan University. The edible portion was cleaned by rinsing several times in tap water and then blended in a cycle blender (Osterizer) with 2 parts (1:2) of cold (4 °C) distilled water for 1 min and then kept stirring overnight in a chilling room (4−5 °C) by a magnetic stirrer. After centrifugation (12000*g*, 4 °C, 30 min), the obtained supernatant was freeze-dried for the convenience of storage and quantification for the following treatments. Subsequently, dissolved CWE (3 mL, 10 mg/mL) was fractionated by a Sephadex G-50 (molecular weight range = 1.5−30 kDa) gel permeation chromatograph (GPC) (column size, 1.6 cm × 85 cm; eluent, distilled water; fractionation, 5 mL/tube) to pool the fraction 1 (F1) (tube 10-16) and fraction 2 (F2) (tube 30-40) monitored at 280 nm. Freeze-dried powders of CWE, F1, and F2 were dissolved and dialyzed against phosphate-buffered saline (PBS; 8 g of NaCl/1.15 g of Na_2HPO_4/0.2 g of KH_2PO_4/0.2 g of KCl/L) prior to use.

Preparation of Conditioned Media (CM). Human peripheral blood was obtained from three normal adult volunteers (aged between 22 and 25) with informed consent, and mononuclear cells (MNC) of each person were separated by density centrifugation (400*g*, 30 min) in a Ficoll-Hypaque solution (1.077 g/mL) (*10*). To 2 mL of MNC (1.5×10^6 cells/mL), which was incubated in 1% glutamine/10% FCS/RPMI 1640 medium, was added 10 μL of CWE (10−800 μg/mL of medium) or of F1 and F2 (200 μg/mL of medium) in PBS. The CM from CWE-stimulated blood MNC (MNC-CM) was collected after incubation at 37 °C in a humidified 5% CO_2 incubator for 1 day (MNC-CM-1) or 5 days (MNC-CM-5). Filtration was conducted through a 0.45 μm membrane prior to storage at −80 °C until use. PHA (5 μg/mL of medium) and PBS were also used to prepare MNC-CM of PHA (PHA-MNC-CM) (positive group) and of PBS (PBS-MNC-CM) (PBS group), respectively. To rule out the possible LPS contamination of MNC-CM samples, polymycin B (100 μg/mL) was added to the sample to prepare the MNC-CM.

Cell Culture and Treatments. U937 cells were cultured in 1% glutamine/10% FCS/RPMI 1640 medium and maintained in an exponential growth status. The cells were incubated in 35 mm Petri dishes at an initial concentration of 1×10^5 cells/mL in the presence or absence (control group) of 20% (v/v) MNC-CM-1 or -5 (MNC-CM method). Adherent cells in day 5 cultures were collected by gently rubbing the dishes with a rubber policeman, and the cell numbers were counted using the Trypan Blue Dye exclusion test (*13*) to determine the growth inhibition (%) (*10*): growth inhibition (%) = (1 − cell number of MNC-CM treatment/cell number of control group) × 100%. In contrast to the MNC-CM method, incubation of U937 cells in the presence of CWE in culturing medium was termed the direct method. Three separate experiments were each tested in duplicate.

Assay for Superoxide Production. The NBT test was applied to detect the production of cytoplasmic superoxide by the differentiated myeloid cells. Cells collected from day 5 cultures were suspended in RPMI medium at a concentration of 1×10^6 cells/mL and then mixed with an equal volume of NBT test stock solution (2 mg of NBT/1 μM phorbol myristate acetate/mL of PBS). After incubation at 37 °C for 30 min, the suspended cells (80 μL) were cytocentrifuged onto glass slides with the aid of a Cytospin (1200 rpm, 5 min) and then counterstained with 0.5% Safranin solution. The percentage of formazan-containing cells was assessed of 200 cells. Three separate experiments were each tested in duplicate.

NSE Determination. Cells collected from day 5 cultures were suspended in RPMI medium at a concentration of 1×10^6 cells/mL and then cytocentrifuged (80 μL) onto glass slides with the aid of a Cytospin (1200 rpm, 5 min). The production of cytoplasmic NSE by the differentiated monocytic cells was detected using a commercially available staining kit based on the formation of brownish black granules in the cytoplasm, resulting from the combination of diazolium salt with naphthol compounds, which were converted from α-naphthyl acetate in the presence of NSE (*14*). The percentage of NSE positive cells was assessed microscopically of 200 cells. Three separate experiments were each tested in duplicate.

Assay for Differentiation Antigens. The expression of monocyte-associated antigens on the surfaces of U937 cells after the induction of differentiation was detected according to the methods described by Goyert et al. (*15*) and Wang et al. (*9*) with minor modifications. To cell suspensions (100 μL) at a concentration of 1×10^6 cells/mL from day 5 cultures was added 2.5 μL of primary mouse anti-human CD11b, CD14, or CD68 antibody. After reaction at 4 °C for 30 min, cells were rinsed twice with 5% FBS/PBS by centrifugation (200*g*, 10 min, 4 °C) and then reacted with 100 μL of 50-fold-diluted FITC-conjugated goat IgG fraction to mouse IgG for 30 min in a dark and cold (4 °C) place. Then, 10 μL of cell suspensions on a microscope glass slide was examined under a light microscope (Eclipse E400, Nikon, Tokyo, Japan) with a fluorescence attachment (Y-FL EPI-fluorescence attachment, Nikon, Tokyo, Japan) at an excitation wavelength of 450−490 nm. CD positive percentage was scored of 100 cells. Three separate experiments were each tested in duplicate.

Cytokine Assay. TNF-α and IL-1β in the various preparations of MNC-CM were quantified separately with the commercial kit by a solid-phase ELISA at a wavelength of 450 nm, according to the method described by Wang et al. (*9*). The correlation coefficients (r^2) for the standard curve of IL-1β (0−160 pg/mL) and of TNF-α (0−500 pg/mL) were 0.998 and 0.999, respectively. MNC-CM was 2-fold-diluted with PBS before assay. Three separate experiments were each tested in duplicate.

Determination of Protein. Crude proteins in CWE and pooled fractions of Sephadex G-50 chromatography were determined according to the Bradford (*16*) method using the Bio-Rad protein assay dye reagent. Bovine serum albumin (0.05−0.5 mg/mL) was used to construct the standard curve ($r^2 = 0.999$). Triplicate samples each were analyzed twice.

Statistical Analysis. Results in tables are presented as mean ± SEM. Differences between the different treatment groups were assessed by Student's test. Data were initially analyzed by one-way analysis of variance, and comparison of groups was made using the Bonferroni multiple-comparison test. A confidence level of 5% (p value of <0.05) was considered to be significant.

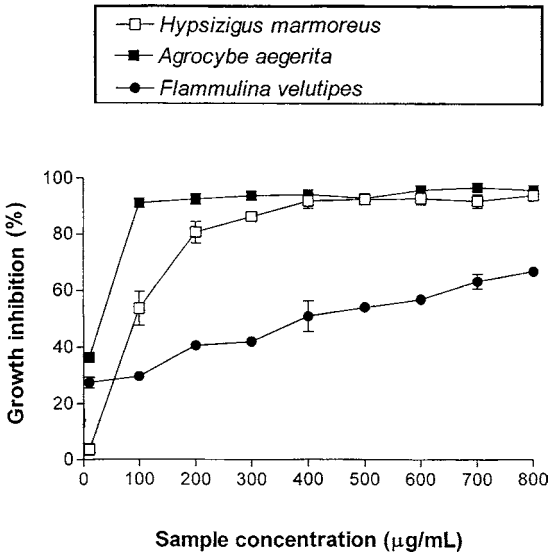

Figure 1. Growth inhibition (percent) of U937 cells incubated with MNC-CM-1 prepared with various levels (10–800 μg/mL) of cold-water extracts from mushrooms. Cells were incubated in the presence of 20% MNC-CM-1, and viable cells were counted after 5 days of cultivation. Bars represent the standard error of means from three separate experiments.

RESULTS

Growth Inhibition of U937 Cells by MNC-CM Prepared with Mushrooms. Figure 1 represents the growth inhibition of U937 cells incubated with MNC-CM-1 (20%, v/v) (MNC-CM method) prepared with 10−800 μg/mL of CWE from mushrooms. Growth of U937 cells was apparently inhibited in a dose-dependent manner. Among the mushrooms tested, AA appeared to be the most potent in inhibiting the myeloid cell growth examined with the MNC-CM method. AA exhibited a sharp increase (from about 40 to 90%) in growth inhibition on tumor cells when CWE increased from 10 to 100 μg/mL. Growth inhibition of ∼50% was observed when 100 μg/mL of HM was used; however, it increased to about 80 and 90% when 200 or 400 μg/mL of CWE from HM was added in culturing medium of MNC, respectively. FV showed mild antitumor effect. In contrast, direct incubation of CWE at the corresponding level (10−400 μg/mL) with U937 cells showed unapparent growth inhibition on tumor cells (data not shown).

CWEs from mushrooms were fractionated by a Sephadex G-50 GPC and the collected high molecular weight protein fraction (F1) (Vo) (MW ≥ 30 kDa) and low molecular weight protein fraction (F2), both of which reached the baseline separation, were pooled to test their growth inhibitory effect on U937 cells (**Table 1**). The chromatograms (data not shown) of HM, AA, and FV on Sephadex G-50 were very similar. It was observed that MNC-CM prepared with F1 of HM (HM1) and AA (AA1) exhibited strong growth inhibition of about 86−88 and 83%, respectively, much higher than that (about 37 and 41%) prepared with F2 of the corresponding mushroom at 200 μg/mL (**Table 1**). With respect to FV, MNC-CM prepared with F1 and F2 showed growth inhibition of 27 and 51% for MNC-CM-1, respectively, revealing that the low molecular weight protein fraction was potent in antitumor activity. In addition, MNC-CM-1 appeared to be stronger in inhibiting tumor growth than MNC-CM-5 prepared with the corresponding mushroom ingredient. Therefore, the active components of mushrooms varied with species and molecular weights.

NBT Test. Table 2 shows the NBT positive (**Figure 2**) percentages of U937 cells incubated with MNC-CM-1 or MNC-CM-5 prepared with fractions of mushroom proteins. MNC-CM-1 prepared with HM1 and AA1 showed higher NBT positive percentage than that prepared with F2 of the corresponding mushroom. Among the fractions and mushrooms tested in the preparation of MNC-CM-1, AA1 was observed to be most potent (∼80%), followed by HM1 (∼68%) and FV2 (∼24%) in inducing the leukemic U937 cells into differentiated monocytes/macrophages with high superoxide production (**Table 2**). In contrast, PHA-MNC-CM-1 showed low NBT positive percentage, whereas PBS-MNC-CM did not show any differentiation-inducing effect. Similar to the results in **Table 1**, MNC-CM-1 appeared to be more effective in inducing the differentiation of U937 cells into monocytes/macrophages by presenting a higher NBT positive percentage than that of MNC-CM-5.

NSE Test. When the MNC-CM-treated U937 cells were subjected to the nonspecific esterase test, remarkable brownish-black granules in the cytoplasm were observed (**Figure 2**), as compared to the PBS-MNC-CM group (4.6%) (**Table 3**). Of note, HM1-stimulated MNC-CM displayed the most marked

Table 1. Comparison of Growth Inhibition of U937 Cells in Day 5 Cultures Incubated with MNC-CM-1 or -5 Prepared with Various Pooled Fractions of Dietary Mushrooms from Sephadex G-50 Chromatography[a]

pooled fraction (200 μg/mL)	growth inhibition[b] (%)	
	MNC-CM-1[c]	MNC-CM-5[c]
HM1[d]	88.1 ± 3.6a	85.6 ± 0.3a
HM2	36.5 ± 5.2a	20.5 ± 1.7b
AA1	83.4 ± 1.3a	82.6 ± 4.9a
AA2	41.2 ± 3.0a	31.9 ± 2.3b
FV1	27.0 ± 2.9a	25.9 ± 2.1a
FV2	51.0 ± 3.0a	44.6 ± 3.6b
PHA[e]	43.8 ± 3.9a	36.6 ± 2.2b
PBS[e]	1.3 ± 0.2a	2.0 ± 0.8a

[a] Results from three separate experiments are expressed as mean ± SEM. Values in the same rows having different letters are significantly different ($p < 0.05$). [b] Growth inhibition (%) = (1 − cell number of MNC-CM treatment/cell number of control group) × 100%. [c] MNC-CM was prepared with 200 μg/mL of pooled fraction from Sephadex G-50 chromatography after incubation with MNC for 1 day (MNC-CM-1) or 5 days (MNC-CM-5). [d] HM1, AA1, FV1: F1 of the corresponding mushroom; HM2, AA2, FV2: F2 of the corresponding mushroom. [e] PHA-MNC-CM, positive group (5 μg/mL). [f] PBS-MNC-CM, PBS group.

Table 2. Comparison of NBT Positive Percentage of U937 Cells in Day 5 Cultures Incubated with MNC-CM-1 or -5 Prepared with Various Pooled Fractions of Dietary Mushrooms from Sephadex G-50 Chromatography[a]

pooled fraction (200 μg/mL)	NBT positive (%)	
	MNC-CM-1[b]	MNC-CM-5[b]
HM1[c]	67.5 ± 7.5a	32.5 ± 0.5b
HM2	16.2 ± 1.2a	14.0 ± 2.0b
AA1	79.7 ± 1.7a	31.7 ± 0.7b
AA2	23.2 ± 0.2a	15.7 ± 1.7b
FV1	17.2 ± 0.7a	6.7 ± 0.2a
FV2	24.2 ± 1.7a	14.5 ± 1.0b
PHA[d]	28.5 ± 8.5a	10.0 ± 2.0b
PBS[e]	0.7 ± 0.2a	0a

[a] Results from three separate experiments are expressed as mean ± SEM. Values in the same rows having different letters are significantly different ($p < 0.05$). [b] MNC-CM was prepared with 200 μg/mL of pooled fraction from Sephadex G-50 chromatography after incubation with MNC for 1 day (MNC-CM-1) or 5 days (MNC-CM-5). [c] HM1, AA1, FV1: F1 of the corresponding mushroom; HM2, AA2, FV2: F2 of the corresponding mushroom. [d] PHA-MNC-CM, positive group (5 μg/mL). [e] PBS-MNC-CM, PBS group

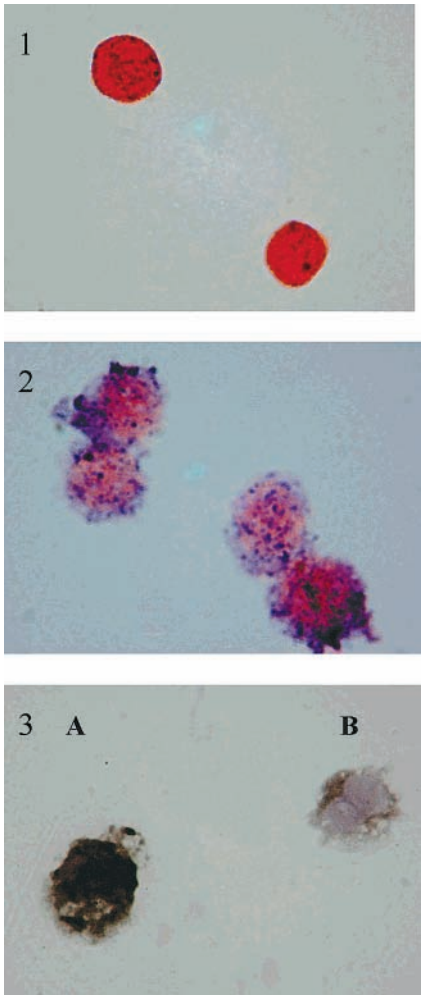

Figure 2. NBT reduction test of undifferentiated (**1**) and differentiated (**2**) cells and nonspecific esterase stain (**3**) of differentiated (**A**) and undifferentiated (**B**) cells. U937 cells were observed under a light microscope (1000×). (Figure is reproduced here at 80% of the original size.)

Table 3. Comparison of NSE Positive Percentage of U937 Cells in Day 5 Cultures Incubated with MNC-CM-1 or -5 Prepared with Various Pooled Fractions of Dietary Mushrooms from Sephadex G-50 Chromatography[a]

pooled fraction (200 μg/mL)	NSE positive (%)	
	MNC-CM-1[b]	MNC-CM-5[b]
HM1[c]	58.6 ± 1.6a	34.6 ± 2.6a
HM2	18.0 ± 1.0a	8.0 ± 0.7b
AA1	47.3 ± 0.6a	26.3 ± 0.3a
AA2	26.3 ± 0.3a	15.3 ± 0.6b
FV1	21.0 ± 1.0a	11.6 ± 1.6a
FV2	33.0 ± 0.3a	23.3 ± 0.6b
PHA[d]	34.0 ± 2.0a	16.6 ± 0.6b
PBS[e]	4.6 ± 0.3a	4.3 ± 0.3a

[a] Results from three separate experiments are expressed as mean ± SEM. Values in the same rows having different letters are significantly different ($p < 0.05$). [b] MNC-CM was prepared with 200 μg/mL of pooled fraction from Sephadex G-50 chromatography after incubation with MNC for 1 day (MNC-CM-1) or 5 days (MNC-CM-5). [c] HM1, AA1, FV1: F1 of the corresponding mushroom; HM2, AA2, FV2: F2 of the corresponding mushroom. [d] PHA-MNC-CM, positive group (5 μg/mL). [e] PBS-MNC-CM, PBS group

NSE positive percentage, either in MNC-CM-1 (58.6%) or in MNC-CM-5 (34.6%), followed by AA1, FV2, AA2, FV1, and HM2. A higher NSE positive percentage suggests the more

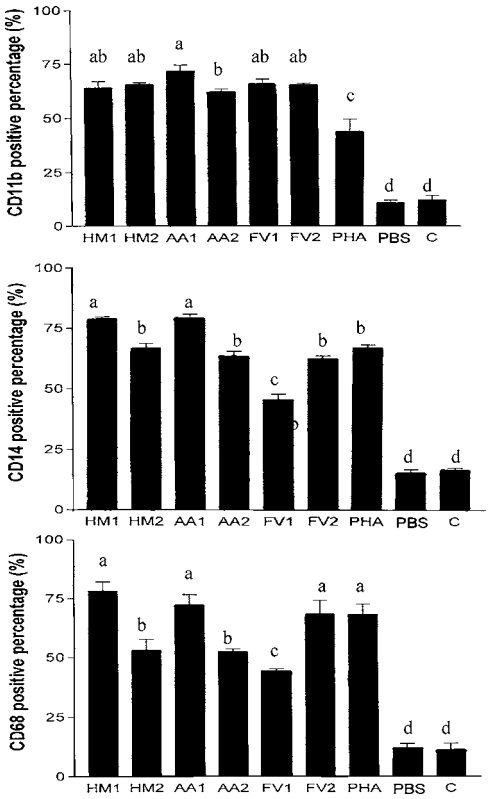

Figure 3. Expressions of monocyte-associated antigens on U937 cells incubated for 5 days in the presence of 20% MNC-CM-1 prepared with 200 μg/mL F1 and F2 fractions of mushrooms on Sephadex G-50 chromatography. Incubated U937 cells were treated with FITC-conjugated goat anti-mouse antibody using a solid-phase ELISA method. HM1, AA1, FV1, F1 of the corresponding mushroom; HM2, AA2, FV2, F2 of the corresponding mushroom; PHA, PHA-MNC-CM, positive group (5 μg/mL medium); PBS, PBS-MNC-CM group; C, control group. Bars represent standard error of means from three separate experiments. Values having different letters are significantly different ($p < 0.05$).

remarkable formation of NSE, which is usually detected in differentiated monocytes/macrophages (*14*). Again, MNC-CM-1 appeared to show a stronger differentiation-inducing effect than MNC-CM-5, as is also indicated in **Table 2**.

Monocyte-Associated Antigens. In **Figure 3**, CD11b positive percentages were all between 60 and 70% when U937 cells were incubated with MNC-CM-1 prepared with 200 μg/mL of protein fractions from mushrooms, compared to only 14% in the PBS-MNC-CM group and 16% in the control group. In addition, high positive percentages of CD14 and CD68 in the day 5 cultures of U937 cells incubated with various preparations of MNC-CM-1 were also determined (**Figure 3**). The CD14 and CD68 positive percentages of differentiated U937 cells induced by HM1 and AA1 were ~75%, suggesting that high molecular weight protein fractions from HM and AA enhanced remarkably the maturation of U937 cells.

Assay for Cytokines in MNC-CM-1 or -5. The TNF-α level in MNC-CM-1 was detected to be 1350 and 892 pg/mL when MNC was stimulated with 200 μg/mL of HM1 and HM2 (**Table 4**), respectively, suggesting the strong tumor necrosis-inducing activity of HM. However, the level of TNF-α remarkably decreased to 736 pg/mL for HM1 and 549 pg/mL for HM2 in MNC-CM-5 during the prolonged incubation. Similar results were observed when F1 was used to prepare MNC-CM-1 and -5 (**Table 4**). With respect to FV, F2 of these mushrooms appeared to be more potent in inducing TNF-α production than F1 (**Table 4**). However, similar to the results for HM and AA,

Table 4. TNF-α and IL-1 β Levels in MNC-CM Prepared with Various Pooled Fractions of Dietary Mushrooms from Sephadex G-50 Chromatography[a]

pooled fraction (200 μg/mL)	TNF-α (pg/mL)		IL-1 β (pg/mL)	
	MNC-CM-1[b]	MNC-CM-5[b]	MNC-CM-1[b]	MNC-CM-5[b]
HM1[c]	1350.0 ± 50.0a	736.3 ± 32.5b	1374.1 ± 55.9a	576.4 ± 30.0b
HM2	891.9 ± 3.1a	549.4 ± 16.9b	858.6 ± 32.3a	323.3 ± 19.4b
AA1	1170.0 ± 10.3a	776.3 ± 31.3b	725.0 ± 25.1a	523.6 ± 22.2b
AA2	567.5 ± 36.3a	295.0 ± 21.3a	584.1 ± 30.9a	314.5 ± 15.9b
FV1	398.1 ± 55.6a	31.4 ± 13.6a	399.7 ± 21.2a	76.3 ± 7.9b
FV2	1198.1 ± 51.9a	523.1 ± 29.4b	992.7 ± 45.5a	284.2 ± 11.2b
PHA[d]	1560.0 ± 60.0a	388.3 ± 21.8b	216.1 ± 17.5a	122.1 ± 8.1b
PBS[e]	6.0 ± 2.5a	0a	0a	0a

[a] Results from three separate experiments are expressed as mean ± SEM. Values in the same rows having different letters are significantly different ($p < 0.05$). [b] MNC-CM was prepared with 200 μg/mL of pooled fraction from Sephadex G-50 chromatography after incubation with MNC for 1 day (MNC-CM-1) or 5 days (MNC-CM-5). [c] HM1, AA1, FV1: F1 of the corresponding mushroom; HM2, AA2, FV2: F2 of the corresponding mushroom. [d] PHA-MNC-CM, positive group (5 μg/mL). [e] PBS-MNC-CM, PBS group

the quantity of TNF-α decreased markedly when the incubation time of MNC with pooled fractions was extended to 5 days.

In addition, results in **Table 4** show that MNC-CM-1 prepared with HM1 contained the highest level of IL-1β (1374 pg/mL), followed by those prepared with FV2 and AA1. PHA exhibited a relatively weak stimulatory effect on MNC to produce this cytokine (216 pg/mL). Increase in the incubation time of pooled fractions of CWE with MNC to 5 days appeared to be unfavorable for the production of IL-1β in MNC-CM (**Table 4**), as in the case of TNF-α.

DISCUSSION

The MNC-CM method was used to observe the suppression of tumor cells by the immunomodulatory components from fungi through the activation of MNC (*9*). Chen et al. (*10*) indicated that the polysaccharide-rich fraction of *Cordyceps sinensis* (10 μg/mL)-stimulated MNC-CM markedly inhibited U937 cell growth by ∼75%, whereas the direct method showed unapparent growth inhibition. Similarly, the polysaccharide-rich fraction of black soybean also exhibited remarkable inhibition on U937 growth by ∼97% with the MNC-CM method (*11*). This suggests the advantages of the MNC-CM method in observing the effect of samples on U937 cell growth over the direct method. Therefore, to evaluate the antiproliferative effect of dietary mushrooms on U937 cells, various preparations (10−800 μg of CWE/mL) of MNC-CM-1 were used. Of note, the presence of 100 μg/mL of CWE from AA in MNC-CM-1 displayed a marked antitumor effect, showing greater effect than that reported by Chen et al. (*10*) at the same level of purified polysaccharide fraction from costly *C. sinensis*. The mechanisms of the activation of MNC by the bioactive ingredients in food and vegetables and the resulted marked antitumor effect (**Figure 1**) were relevant to the enhancement on cell-mediated immunity in humans (*9*, *10*). Such results were firmly supported by the use of polymyxin B added in the sample to prepare MNC-CM in order to clarify that the antitumor effect was due to sample, rather than the presence of LPS in CWE.

Macrophages produce superoxide through the respiratory burst response as the results of the reactions of oxygen with nicotinamide-adenine dinucleotide phosphate (NADPH) to form superoxide anion and the following reactions with superoxide dismutase (SOD). Accordingly, stronger superoxide production revealed a more remarkable bactericidal effect induced by the MNC-CM method (**Table 2**) (*9*, *17*). The polysaccharide-rich fraction (10 μg/mL) of *C. sinensis* in MNC-CM was recently reported to be potent in differentiating U937 cells and showed ∼90% NBT positive percentage (*10*). In addition, Liao et al. (*11*) indicated that incubation with MNC-CM prepared with 400 μg/mL soybean polysaccharides increased the NBT positive percentage of U937 cells to ∼97%, whereas an unapparent NBT positive percentage was observed in the direct treatment of cells. However, in contrast to the MNC-CM method, the presence of 5 nM bufalin (*18*) and 20 μM geranylgeranylacetone (*19*) in the culturing medium (direct method) also led to 92 and 41% NBT positive percentage of human myeloid leukemia cell lines, respectively.

Maturation and differentiation of U937 cells were also observed by using the NSE method (**Figure 2**). Detection of the produced cytoplasmic NSE is more specific than chloroacetate esterase (CAE) in differentiating monocytes (*14*), and thus, the formation of brownish-black granules in the cytoplasm is one of the features in differentiated monocytes/macrophages. In the present study, HM1 (200 μg/mL) in MNC-CM-1 induced ∼58.6% of NSE positive percentage (**Table 3**), slightly higher than the (∼50%) of NSE positive percentage in U937 cells induced by polysaccharide-rich fraction stimulated MNC-CM (*10*).

CD14 and CD68 are usually detected in monocytes and macrophages (*15*), whereas CD11b is one of the main antigen expressions in monocytes, granulocytes, and natural killer (NK) cells (*20*). Increases in these antigen expressions reveal the differentiation induction by mushroom-stimulated MNC-CM. The CD11b positive percentage (∼65%) of U937 cells induced by MNC-CM-1 prepared with F1 or F2 of CWE for 5 days appeared to be higher than that (∼45%) of cells induced by polysaccharide fraction from costly *G. lucidum* (*17*) or *C. sinensis* (*10*) when detected by a FITC-conjugated goat anti-mouse antibody using an ELISA. Similar results were observed in CD14 and CD68 positive percentage groups, revealing the potent differentiating-inducing effects of U937 cells by protein-rich fractions from dietary mushrooms.

TNF-α is a polypeptide of 17 kDa (152 amino acids) produced by activated T- and B-lymphocytes, neutrophils, and NK cells (*21*) and is a potent tool for tumor cell necrosis (*22*). IL-1β, a polypeptide of ∼17 kDa, is usually produced by monocytes and macrophages. It is capable of activating T- and B-lymphocytes and of inducing the production of GM-CSF from peripheral blood lymphocytes (*23*, *24*). In addition, it displays cytotoxicity and antiproliferation on tumors as the results of the release of TNFs and interferons by stimulating macrophages and lymphocytes (*24*). Therefore, the presence of mediators released from MNC upon stimulation by mushroom proteins appeared to be important in inducing the differentiation of myeloid leukemic U937 cells (*9*). MNC-CM prepared with 200 μg/mL polysaccharides from *G. lucidum* contained ∼1100 pg/mL IL-1β (*9*), slightly lower than that (1374 pg/mL) prepared with HM1 but higher than that (993 pg/mL) prepared with FV2 at the same dosage (**Table 4**). Similarly, the activity of TNF-α in MNC-CM-1 prepared with HM1, AA1, or FV2 was close to that (1200 pg/mL) in MNC-CM prepared with 100 μg/mL polysaccharides from *G. lucidum* (*9*, *17*). This suggests that CM from protein-stimulated MNC contains some cytokines that help to induce the differentiation of U937 cells and the increases of the expressions of monocyte-associated antigens. However, an apparent reduction in cytokine level was observed in the prolonged incubation during the preparation of MNC-CM.

In summary, CWE from AA, HM, or FV showed strong

growth inhibitory effect on U937 cells by using the MNC-CM method, and all were found to be potent monocyte-associated cytokine inducers that promoted the production of TNF-α and IL-1β from human blood peripheral MNC. This reveals that those mushrooms enhance the cell-mediated immunity through the activation of T- and B-lymphocytes and macrophages and appear to be immunomodulators. In addition, high percentages of differentiated U937 cells were presented by tests of NBT, NSE, and CD markers after incubation with protein-stimulated MNC-CM. Fractions in CWE of each mushroom displayed varied antitumor activities, suggesting that the level of antitumor mediator or differentiation-inducing factor was dependent on the mushroom source. Compared to MNC-CM-5, MNC-CM-1 appeared to be more effective in antitumor and differentiation-inducing activities due to the higher cytokine contents. Antitumor tests of purified ingredients from AA, HM, or FV in vivo appeared to be of interest.

LITERATURE CITED

(1) Jong, S. C.; Birmingham, J. M. Medicinal therapeutuc value of the Shitake mushroom. *Adv. Appl. Microbiol.* **1993**, *39*, 153−184.

(2) Ko, J. L.; Hsu, C. I.; Lin, R. H.; Kao, C. L.; Lin, J. Y. A new fungal immunomodulatory protein, FIP-fve isolated from the edible complete amino acid sequence. *Eur. J. Biochem.* **1995**, *228*, 244−249.

(3) Wasser, S. P.; Weis, A. L. Therapeutic effects of substances occurring in higher Basidiomycetes mushroom: a modern perspective. *Crit. Rev. Immunol.* **1999**, *19*, 65−96.

(4) Leung, M. Y.; Fung, K. P.; Choy, Y. M. The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. *Immunopharmacology* **1997**, *35*, 255−263.

(5) Ikekawa, T.; Ikeda, Y.; Yoshioka, Y.; Nakanishi, K.; Yokoyama, E.; Yamazaki, E. Studies on antitumor polysaccharides of *Flammulina velutipes* Sing. II. The structure of EA3 further purification of EA5. *J. Pharmacobio-Dyn.* **1982**, *5*, 576−581.

(6) Ikekawa, T.; Maruyama, H.; Miyano, T.; Okura, A.; Sawasaki, Y.; Naito, K.; Kawamura, K.; Shiratori, K. Proflamin, a new antitumor agent: preparation, physiochemical properties antitumor activity. *Jpn J. Cancer Res.* **1985**, *76*, 142−148.

(7) Wang, H.; Gao, J.; Ng, T. B. A new lectin with highly potent antihepatoma and antisarcoma activities from the oyster mushroom *Pleurotus ostreatus*. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 810−816.

(8) Sakagami, H.; Sugaya, K.; Utsumi, A.; Fujinaga, S.; Sato, T.; Takeda, M. Stimulation by PSK of interleukin-1 production by human peripheral blood mononuclear cells. *Anticancer Res.* **1993**, *13*, 671−676.

(9) Wang, S. Y.; Hsu, M. L.; Hsu, H. C.; Tzeng, C. H.; Lee, S. S.; Shiao, M. S.; Ho, C. K. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages T-lymphocytes. *Int. J. Cancer* **1997**, *70*, 699−705.

(10) Chen, Y. J.; Shiao, M. S.; Lee, S. S.; Wang, S. Y. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. *Life Sci.* **1997**, *60*, 2349−2359.

(11) Liao, H. F.; Chou, C. J.; Wu, S. H.; Khoo, K. H.; Chen, C. F.; Wang, S. Y. Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U937 cells. *Anti-Cancer Drugs* **2001**, *12*, 841−846.

(12) Chen, Y. Y.; Chang, H. M. Antiproliferative and differentiating effects of polysaccharide fraction from Fu-Ling (*Poria cocos*) on human leukemic U937 and HL-60 cells. *Food Chem. Toxicol* **2004**, *42*, 759−769.

(13) Zweier, J. K.; Kuppusamy, P.; Lutty, G. A. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4046−4050.

(14) Yam, L. T.; Li, C. Y.; Crosby, W. H. Cytochemical identification of monocytes and granules. *Am. J. Clin. Pathol.* **1971**, *55*, 283−290.

(15) Goyert, S. M.; Ferreo, E. M.; Seremetis, S. V.; Winchester, R. J.; Silver, J.; Mattison, A. C. Biochemistry expression of myelomonocytic antigens. *J. Immunol.* **1986**, *137*, 3909−3914.

(16) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(17) Lieu, C. W.; Lee, S. S.; Wang, S. Y. The effect of *Ganoderma lucidum* on induction of differentiation in leukemic U937 cells. *Anticancer Res.* **1992**, *12*, 1211−1216.

(18) Zhang, L.; Nakaya, K.; Yoshida, T.; Kuroiwa, Y. Bufalin as a potent inducer of differentiation of human myeloid leukemia cells. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 686−693.

(19) Sakai, I.; Tanaka, T.; Osawa, S.; Hashimoto, S.; Nakaya, K. Geranylgeranylacetone used as an antiulcer agent is a potent inducer of differentiation of various human myeloid leukemia cell lines. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 873−879.

(20) Ault, K. A.; Springer, T. A Cross-reaction of a rat-anti-mouse phagocyte-specific monoclonal activity (anti-mac-1) with human monocytes NK cells. *J. Immunol.* **1981**, *126*, 359−364.

(21) Vilcek, J.; Lee, T. H. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **1991**, *266*, 7313−7316.

(22) Carswell, E. A.; Old, L. J.; Kassel, R. L.; Green, S.; Fiore, N.; Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3666−3670.

(23) Kaushansky, K.; Lin, N.; Adamson, J. W. Interleukine 1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony-stimulating factors. Mechanism for the hematopoietic response to inflammation. *J. Clin. Invest.* **1988**, *81*, 92−97.

(24) Gasson, J. C. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood* **1991**, *77*, 1131−1145.

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